UNIVERSITI PUTRA MALAYSIA

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF YERSINIA SPP FROM MEAT AND MEAT PRODUCTS

AHMAD ZAINURI MOHD. DZOMIR.

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF *YERSINIA* SPP FROM MEAT AND MEAT PRODUCTS

By

AHMAD ZAINURI MOHD. DZOMIR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia
In Fulfilment of the Requirement for the Degree of Doctor of Philosophy

May 2005

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF *YERSINIA* SPP FROM MEAT AND MEAT PRODUCTS

By

AHMAD ZAINURI MOHD. DZOMIR

May 2005

Chairman: Professor Gulam Rusul Rahmat Ali, PhD

Faculty: Food Science and Technology

Three hundred and twenty two samples comprising of beef (94), chicken parts (114), pork (11), beef burger (47), chicken burger (30), chicken nugget (1), chicken frankfurter (10), chicken carcass (20) and pork frankfurter (5) were examined for the presence of *Yersinia*. Samples were enriched in phosphate-buffered-saline at 25°C for 48h. Enriched samples were treated with 0.5% potassium hydroxide (KOH) solution and then streaked onto Cefsulodin-Irgasan-Novobiocin (CIN) agar plates. 1/94 (1.1%), 16/47 (34.0%), 6/14 (5.3%), 1/30 (3.3%) and 1/20 (5.0%) of beef, beef burgers, chicken parts, chicken burgers and chicken carcass samples were contaminated with *Yersinia* spp. respectively. *Yersinia* spp. were not isolated from pork, chicken nugget and chicken frankfurter samples. Fifty-three

isolates of *Yersinia* spp. were isolated from 25 (7.7%) positive samples and identified as *Y. enterocolitica* (29), *Y. frederiksenii* (18), *Y. kristensenii* (3) and *Y. intermedia* (3). Highest numbers of positive samples were obtained from Selangor (86.2%), followed by Negeri Sembilan (6.9%). 3.4% of the positive samples were obtained from Kuala Lumpur and overseas. In this study, *Y. enterocolitica* was defined as non sensu stricto on the basis of biochemical properties not strictly fitting according to the scheme used for classification at the genus level. They were biochemically atypical, including Simmon's citrate-positive and Voges-Proskauer-negative. All *Y. enterocolitica* isolates were grouped into biotype 1A based on reaction to D-xylose, nitrate reduction and pyrazinamidase. The four related species: *Y. frederiksenii*, *Y. intermedia* and *Y. kristensenii* were readily distinguishable by sucrose, melibiose, rhamnose and raffinose fermentation. The result of serotyping of twenty *Y. enterocolitica* showed that eleven of them belonged to serotype O:52,53; one isolate belong to serotype O:41,42 and nine were untypable, delineating the isolates from pathogenic serotypes. In addition, Polymerase Chain Reaction (PCR) analysis indicated that *Y. enterocolitica* examined did not possessed any of the virulence marker genes that are characteristics of pathogenic strains. Antibiotic susceptibility analysis showed there was no difference in the susceptibilities of the four *Yersinia* species towards ampicillin, penicilin, cephalotin, bacitracin and chloramphanicol. All isolates (100%) were resistant to ampicilin, penicilin and cephalotin but all (100%) were sensitive to chloramphenicol. 1.96, 3.92, 5.88, 9.80 and 29.41%

of *Yersinia* isolates were resistant to gentamicin, nalidixic acid, streptomycin, tetracycline and cefaporazone, respectively. 98.04% of the isolates which were resistant to carbenicillin demonstrated weak activity of carbenicilin against the *Yersinia*. Multiple Antibiotic Resistance (MAR) index ranged from 0.36 to 0.64. Three plasmid patterns were observed among the *Yersinia* isolated. 31 (60.78%) of the isolates carried single plasmid and 20 (39.22%) of the isolates did not carry any plasmid. 21 (41.18%), 8 (15.69%) and 2 (3.92%) of the isolates were harbor 54, 32 and 2.7 MDal plasmid size, respectively. The dendrogram obtained by comparative analysis of the pulsed field electrophoresis patterns clustered biotype 1A into three clusters (A, B and C). The PFGE result indicated that they (biotype 1A) were different from the pathogenic strains (control strains).

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMENCILAN, PENGENALPASTIAN DAN PENCIRIAN *YERSINIA* SPP. DARIPADA DAGING DAN PRODUK DAGING

Oleh

AHMAD ZAINURI MOHD. DZOMIR

Mei 2005

Pengerusi: Profesor Gulam Rusul Rahmat Ali, PhD

Fakulti: Sains dan Teknologi Makanan

Tiga ratus tiga puluh dua sampel daging lembu (94), daging ayam (114), daging khinzir (11), burger lembu (47), burger ayam (30), nugget ayam (1), frankfurter ayam (10), bilasan daging ayam (20) and frankfurter khinzir (5) telah diperiksa bagi mengesan kehadiran *Yersinia.* Agar cefsulodin-irgasan-novobiocin (CIN) telah diinokulasikan dengan sampel yang telah dinkubasi dalam phosphate-buffered-saline (PBS) 1/15M, pH 7.6 pada suhu 25°C selama 48 jam setelah ia dirawat dengan 0.5% larutan potassium hydroxide (KOH). Frekuensi sampel yang positif adalah seperti berikut; daging lembu (1.1%), burger lembu (34.0%), daging ayam (5.3%), burger ayam (3.3%) dan bilasan daging ayam (5.0%). Daging khinzir, nugget ayam dan frankfurter ayam didapati tidak mengandungi *Yersinia.* Lima puluh tiga kultur Yersinia

telah dipencil dari 25 (7.7%) sampel dan dikenalpasti sebagai *Y. enterocolitica* (29), *Y. frederiksenii* (18), *Y. kristensenii* (3) and *Y. intermedia* (3). Taburan *Yersinia* yang dipencil menurut tempat asal sampel mendapati Selangor (86.2%) sebagai penyumbang utama kepada kontaminasi. Ini diikuti dengan Negeri Sembilan (6.9%) dan 3.4% bagi setiap sampel dari Wilayah Persekutuan dan sampel yang diimpot. *Y. enterocolitica* dalam kajian ini dikatakan *non sensu stricto* berdasarkan ciri-ciri biokimia yang kurang menepati corak klasifikasi bagi tahap genus. Ia menunjukkan ciri-ciri biokimia yang atipikal, termasuk positif bagi ujian sitrat Simmon dan negatif bagi ujian voges-proskauer. Kesemua pencilan *Y. enterocolitica* digolongkan di dalam biotip 1A menurut keputusan ujian-ujian D-xylose, penurunan nitrat dan pyrazinamidase. Keempat-empat spesis *Yersinia*: *Y. frederiksenii*, *Y. intermedia* dan *Y. kristensenii* boleh dibezakan dengan mudah berdasarkan keputusan ujian fermentasi sukrosa, melibiosa, rhamnosa dan raffinosa. Percubaan untuk mengaitkan kadar pemencilan dengan tempoh pemencilan bulanan didapati tidak berjaya. Keputusan ujian serotip bagi dua puluh kultur *Y. enterocolitica* menunjukkan sebelas darinya tergolong dalam serotip O:52,53; satu kultur O:41,42 dan sembilan kultur tidak boleh diserotipkan. Ini menunjukkan mereka tidak tergolong dalam serotip yang virulen. Tambahan pula, analisis PCR menunjukkan kesemua *Y. enterocolitica* yang diuji tidak mempunyai gen virulen seperti yang dipunyai oleh kultur yang patogen. Ujian kerintangan kepada antibiotik menunjukkan tiada perbezaan kerintangan yang ketara ditunjukkan oleh kempat-empat spesis *Yersinia* terhadap

ampicilin, penicilin, cephalotin, bacitracin dan chloramphenicol dimana kesemuanya (100%) rintang terhadap ampicilin, penicilin dan cephalotin. Manakala kesemuanya (100%) adalah sensitif terhadap chloramphenicol. Gentamicin, nalidixic acid, streptomycin, tetracycline dan cefaporazone menunjukkan aktiviti berkesan dimana hanya 1.96, 3.92, 5.88. 9.80 dan 29.41% *Yersinia* yang rintang. Sebanyak 98.04% kerintangan menandakan aktiviti tidak berkesan carbenicilin terhadap *Yersinia*. Analisis indeks MAR yang dihitung menunjukkan julat antara 0.36 hingga 0.64. Tiga corak plasmid diperhatikan: 31 (60.78%) membawa satu plasmid dan 20 (39.22%) tidak mempunyai plasmid. 21 (41.18%), 8 (15.69%) dan 2 (3.92%) masing-masing membawa plasmid bersaiz 54, 32 dan 2.7 MDal. Dendrogram yang diperolehi dari analisis perbandingan corak elektroforesis pulsed-field menggolongkan biotip 1A kepada tiga kumpulan besar (A, B dan C). Keputusan ini menunjukkan bahawa biotip 1A adalah berbeza dari kultur patogenik (kultur kawalan) setelah method PFGE digunakan.

ACKNOWLEDGEMENTS

All praise to Almighty Allah, the Merciful and the Benevolent. Had it not been due to His will and favour, the completion of this study would not have been possible.

I would like to acknowledge the following people for making this study possible.

First I would like to express my sincere appreciation to Prof. Gulam Rusul Rahmat Ali, Prof. Son Radu and Dr. Sahilah Abd Mutalib for their invaluable guidance, advice, supervision and patience in supervising this study.

I would also like to extend my sincere appreciation to Dr. Maznah Ahmad and Dr. Akma Ngah Hamid, Regional Veterinary Diagnostic Laboratory and Dr. Rafal Gierzynski, National Institute of Hygiene, Poland. Thank you as no words can express my gratitude for the help and support.

I also wish to thank Dr. Astrid Waage and Dr. A. Rakin the cultures contributor. I realize that the study could not be completed without the help of Dr. Elisabeth Carniel, Institut Pasteur of Paris who helped me with the serotyping and for that I will always be grateful to Dr. Carniel.

My sincere thanks also goes to members of the laboratory and finally to my family for their belief in me. Their never-ending support gave me the strength to complete the study.

May Allah Bless All of You

AHMAD ZAINURI MOHD. DZOMIR

I certify that an Examination Committee met on 4th May 2005 to conduct the final examination of Ahmad Zainuri Mohd. Dzomir on his Doctor of Philosophy thesis entitled "Isolation, Identification and Characterization of *Yersinia* spp from Meat and Meat Products" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Zaiton Hassan, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Raha Abdul Rahim, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Fatimah Abu Bakar, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Internal Examiner)

Ahmad E. Yousef, PhD
Professor
The Ohio State University
(External Examiner)

ZAKARIAH ABDUL RASHID, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 21 JUL 2005

This thesis is submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor Philosophy. The members of the Supervisory Committee are as follows:

Gulam Rusul Rahmat Ali, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Son Radu, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

Sahilah Abdul Mutalib, PhD
Research Officer
Strategic, Environment, Natural Resources Research Center
Malaysian Agriculture Research and Development Institute
(Member)

AINI IDERIS, PhD
Professor/ Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 11 AUG 2005

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

AHMAD ZAINURI MOHD. DZOMIR

Date: 19 JUL 2005

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

DNA	deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
EDTA	ethylenediamine tetra-acetic acid
EtBr	ethidium bromide
G	Gram
H	hour(s)
HCl	hydrochloric acid
KAc	potassium acetate
KCl	potassium chloride
M	Molarity
MAR	multiple antibiotic resistance
MDa	Megadalton
$MgCl_2$	magnesium chloride
Mg	Milligram
Min	Minutes
Ml	Milliliter
Mm	Millimeter
mM	Millimolar
µg	Microgram
µm	Micrometer
µl	Microliter

Mol	Mole
N	Normal
NaCl	sodium chloride
NaOH	sodium hydroxide
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
R	Resistant
RAPD	random amplification of polymorphic DNA
RNA	ribonucleic acid
Rpm	revolution per minute
rRNA	ribosomal ribonucleic acid
S	Susceptible
subsp.	Subspecies
SDS	sodium dodecyl sulphate
spp.	Species
TBE	tris-borate EDTA electrophoresis buffer
Tris	tris (hydroxymethyl) methylamine
Uv	ultra violet
V	Volts
WHO	World Health Organization
w/v	weight per volume
v/v	volume per volume
>	more than

%	Percentage
°C	degree celcius
P+	with plasmid
P-	without plasmid

CHAPTER 1

INTRODUCTION

Yersinia enterocolitica and *Yersinia enterocolitica*-like bacteria, including *Y. frederiksenii*, *Y. kristensenii*, *Y. intermedia*, *Y. aldovae*, *Y. rohdei*, *Y. mollaretti* and *Y. bercovieri* constitute a fairly heterogeneous group of bacteria which includes both well-established pathogens and range of environmental or non-pathogenic strains. *Yersinia* is a gram negative, non-spore forming, facultative anaerobic bacterium that can multiply at refrigeration temperatures, 0-4°C (Bottone, 1997; Bottone, 1999).

There has been a steady increase in the number of isolations of *Y. enterocolitica* in recent years, not only from clinical materials but also from water and foods. *Y. enterocolitica* and related species are common in many types of foods such as milk (Moustafa *et al.*, 1983; Franzin *et al.*, 1984; Toora *et al.*, 1989; Ibrahim and Rae, 1991), meat and meat products (Logue *et al.*, 1996; Fukushima *et al.*, 1997; Johannessen *et al.*, 2000; Ramirez *et al.*, 2000), also in other animal associated products (Velazquez *et al.*, 1993). *Yersinia* is of particular importance for the safety of consumers, because it is capable of growing in raw meat and meat products and remains viable for long periods at refrigeration temperatures (Hudson and Mott, 1993; Johnson *et al.*, 1982; Myers et *al.*, 1982). As the number of *Yersina* in foods is usually

low and there is often great variety of background flora, direct isolation on selective plating media is seldom successful. Isolation methods usually involve enrichment of the sample followed by plating onto selective agar media and confirmation of typical colonies (Hoorfar and Holmvig, 1999; Johannessen *et al.*, 2000). In addition, alkalotolerance in *Y. enterocolitica* compared to other bacteria has been used to reduce the level of background flora and made detection of *Y. enterocolitica* easier (Jiang *et al.*, 2000).

Unlike *Salmonella* and *Shigella* species, which are intrinsic pathogens and essentially all strains can cause enteric infections, there is strain-to-strain variation in the pathogenicity of *Y. enterocolitica*. A number of studies have shown an excellent correlation between the serotype and biotype of *Y. enterocolitica.* Biotype 1A usually comprises avirulent strains and encompasses a wide range of serotypes (O:5; O:6,30; O:6,31; O:7,8; O:10) as well as non-typable O strains (Tennant *et al.*, 2003). Whereas biotype 1B and biotype 2-5 include strains that are potentially pathogenic for man and animal. These biotype belonged to only a few serotypes such as O:3; O:5,27; O:8; O:9 of *Y. enterocolitica* sensu stricto. An accurate designation of pathogenic *Y. enterocolitica* strains need to take into account both the biotype and the serotype of a strain. The two traits are linked closely. Biotype 4 is associated with serotype O:3 (4/O:3), biotype 2 with serotype O:9 and, less frequently O:5, and biotype 3 with serotype O:3 and O:5. Therefore, the